GEORGIA INSTITUTE OF TECHNOLOGY
Engineering Experiment Station
Atlanta, Georgia

TERMINAL PROGRESS REPORT

PROJECT B-316

RADIATION INDUCED CELL DEATH AT VERY LOW TEMPERATURES

By

R. H. FETNER

Grant in Aid RH00386-02

1 MARCH 1967 to 29 FEBRUARY 1968
Issued 31 August 1968

Prepared for
PUBLIC HEALTH SERVICE
BUREAU OF DISEASE PREVENTION & ENVIRONMENTAL CONTROL
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SUMMARY

Reproductive cell death and chromosome exchanges produced in primary Chinese hamster cells after exposure to x-rays were studied with irradiation performed at room temperature and in liquid nitrogen (-190°C). In the cells irradiated at room temperature, the e^{-1} ($\tilde{D}_o$) was 179 rads and there were .39 chromosome exchanges per cell at this dose. With the irradiations performed in liquid nitrogen, the $\tilde{D}_o$ was 977 rads and there were .21 chromosome exchanges per cell at this dose. Thus, irradiation at the lower temperatures resulted in 38 percent fewer chromosome exchanges per cell and this would support the argument that chromosome exchanges may not be the primary cause of reproduction cell death.

INTRODUCTION

Chromosome exchanges are one of the results of exposure to high energy radiation which have been implicated as an important cause of mammalian reproductive cell death. There are, however, good arguments against this concept and the question remains unresolved. It is difficult to isolate the contribution of one radiation-induced effect, such as chromosome exchanges, from other events which are also present. One approach to this problem is to study both chromosome aberration production and reproductive cell death produced by exposure to high energy radiation under different physical conditions and to determine if the quantitative relationship between the two events is changed.

Widely different temperatures would provide the conditions for such a study and this is a report on the comparative effectiveness of x-rays in producing reproductive cell death and chromosome exchanges with irradiations performed at room temperature and in liquid nitrogen (-190°C).

A number of different mammalian cell types have been successfully employed in radiation survival studies and the general uniformity of the results has been encouraging. Studies of chromosome aberrations, however, are more restrictive of the cell system which may be used. Experience gained on work with plant cells, which comprises a relatively large volume of literature, has emphasized that certain criteria must be imposed if analysis of chromosome exchanges are to be valid. Of primary consideration is the requirement that each metaphase chromosome complement must be analyzed as a unit. All of the centromeres in a metaphase cell must be accounted for before it is possible to verify the presence of chromosome exchanges. For

example, a cell which contains a dicentric chromosome at the first metaphase after its production will still contain the normal number of chromosome bodies, except that one of these bodies will have two centromeres and the other will not -- the acentric fragment. Further, the small size of even the most favorable mammalian chromosomes makes it easy to confuse centromeres and crossed chromatids and the only reliable verification is to analyze the entire chromosome complement. If this elementary requirement is to be met the cell population must have a predictable chromosome number. This precludes the use of established cell lines and makes it desirable to work with material as close to the primary tissue as experimentally feasible.

The experiments reported here were performed with single transfer cell lines from minced 10 day old Chinese hamster embryos.

MATERIALS AND METHODS

The source for the in vitro cell cultures used in this study was ten 12 day old embryos of the Chinese hamster (Cricetulus griseus). Animals used were in the 5-7th inbred generation (brother - sister matings) from animals originally obtained from the colony of Dr. G. Yearganian, Harvard University. First transfer cell lines were used in the cell survival experiments and for the chromosome analysis. The designation "cell line" conforms to the definition suggested by the Committee on Terminology of the Tissue Culture Association. All cells were grown in Eagles essential medium plus non-essential amino acids and twenty percent fetal calf serum (Reheis Chemical Co.). Penicillin and streptomycin were also included. The specific techniques were as follows.

Cell Survival Studies

Ten 12 day old embryos were washed, finely minced and placed in plastic flasks. After 5 hours, the non-adhering tissue fragments were decanted and the media replaced. The media was changed daily and after 5-6 days the resulting outgrowth of cells was scraped from the surface and agitated vigorously. The flasks were placed on end and the cell suspension transferred to fresh flasks by pipettes. Care was taken not to include pieces of tissue in this transfer. After 5-6 days these first transfer cultures contained a confluent fibroblast-appearing cell population. Media was decanted from these cultures and 15 ml of 0.2% trypsin (Difco 1:200) was added. The trypsin solution was made up in Ca and Mg free Hanks solution, the pH adjusted to 8.2 by addition of dilute NaOH and then sterilized by passing through a 0.2 μ millipore filter. Gentle agitation of the trypsin solution was used

to release the cells from the flask surface and then the solution was passed repeatedly through a 27 gauge hypodermic needle until observation under the microscope indicated a single cell suspension. This usually required about 6 passages through the syringe. In this procedure the cell suspension was poured into the syringe, the plunger inserted and the solution forced out through the needle. When a single cell suspension had been achieved, it was diluted into a media composed of 60 percent growth media, 30 percent fetal calf serum, and 10 percent glycerol. The final concentration of cells was adjusted to 50,000-100,000/ml as determined by the Coulter counter. This cell suspension was pipetted into 5 ml ampoules and flame sealed. One half of the ampoules was frozen in a programmed rate freezer (Canalco Co.) and the rest were left at room temperature and used immediately for radiation studies. Ampoules were frozen at 1°/min to -30°C and then were placed immediately in liquid nitrogen. After irradiation these ampoules were rapidly thawed by agitation in warm water and then diluted, plated out and incubated in sealed chamber flushed with 5 percent CO_2. These dishes were incubated undisturbed at 35°C for 12 days, the media decanted and then covered with 3:1 ethanol-acetic acid for 30 minutes. The fixative was removed and the dishes rinsed with 95 percent ethanol and permitted to air dry. The colonies were stained by covering with a saturated solution of 45 percent propionic acid-orcein for 15 minutes, washing with 95 percent alcohol until excess dye was not evident in the alcohol rinse, and then air dried. Enumeration of colonies was performed under a low power disecting microscope against a grid background.

Chromosome Exchange Studies

Cell cultures were obtained from minced embryos as described in the previous section. These primary cultures were harvested by scraping in these experiments and were not exposed to trypsin. After irradiation the ampoules were opened and the contents emptied into large mouth French square bottles which contained a microscope slide. An additional volume of 40-45 ml of media was added and the bottles incubated for 5 hours at which time the media was changed. In all cases the cells had adhered to the slide surfaces by this time. The frozen samples attached as readily as the non-frozen cultures. After 15 hours of culture, colchine was added to produce a final concentration of 2 mg/l, and 5 hours later the cultures were treated with hypotonic media (20 percent strength) for 20 minutes and then the microscope slides fixed in acetic acid-ethanol (1:3) for 15 minutes. These slides were chilled and then flamed dry. Immediately before analysis, these slides were stained with freshly prepared propionic acid-orcein, sealed with sticky wax and observed under phase optics. Fresh preparations give better results than permanent preparations.

Irradiations were performed with a 250 KV x-ray machine at 15 ma and 0.5 mm cu and 1 mm of aluminum filtration. The specimens were 25 cm from the source and gave a half layer value equal to 2 mm of aluminum. The dose rate was determined with a Victoreen thimble and was checked against a Frick dosimeter with the ferric ion yield measured at 224 mμ. The chemical dosimeter was placed in 5 ml ampoules in the same way as the cell samples. The dose rate was 80-85 r/minute in all of the experiments.

RESULTS

In the cell survival experiment the frozen and non-frozen series had a plating efficiency of .29 and .18, respectively. These were determined as a function of total cell counts and no attempt was made to determine cell viability by dye exclusion. No significance can probably be attached to the differences between the two plating efficiencies and the comparison serves to indicate that, if cells are carefully frozen, they respond upon thawing no different from non-frozen cell cultures.

Results of the irradiation at room temperature (RT) are presented in Table I and Figure 1. These data indicated an exponential curve from 100 r on and with a least squares fit the estimating equation was determined to be

$$\log Y = .3340 + .1660 \log X$$

where a and b are negative logarithms, with S log y·x = .0226 and r = .9995. The sample standard deviation of the regression coefficient (Sb) was equal to .0006 and a "t" test indicated that b was highly significant--556 with 4 d.f.. The extrapolation number ($\tilde{n}$) was 2.1 and the dose required to reduce survival by the factor 1/e ($\tilde{D}_o$) was 179 rads. These two parameters agree with reports of similar experiments by others.

When cells were irradiated at liquid nitrogen temperatures (-190°C) (Table II and Figure 1), the slope of the survival curve appears to be truly exponential with no detectable shoulder. The curve does not go through several decades because the highest dose used, 1600 r, only reduced the surviving fraction 80 percent. Even with this limitation there was a good fit to the estimating equation:

$$\log Y = .0391 + .0402 \log x$$

DOSE RADS x 10^2	SURVIVING FRACTION
0	1.00
0.5	.62
1.0	.62
2.0	.31
2.0	.34
4.0	.07
4.0	.07
8.0	.003

TABLE I. Reproductive survival of Chinese hamster cells after various doses of 250 kV x-rays at room temperature

DOSE RADS x 10^2	SURVIVING FRACTION
0	1.00
1.0	.78
2.0	.73
4.0	.63
8.0	.53
16.0	.19

TABLE II. Reproductive survival of Chinese hamster cells after various doses of 250 kV x-rays at liquid nitrogen temperatures

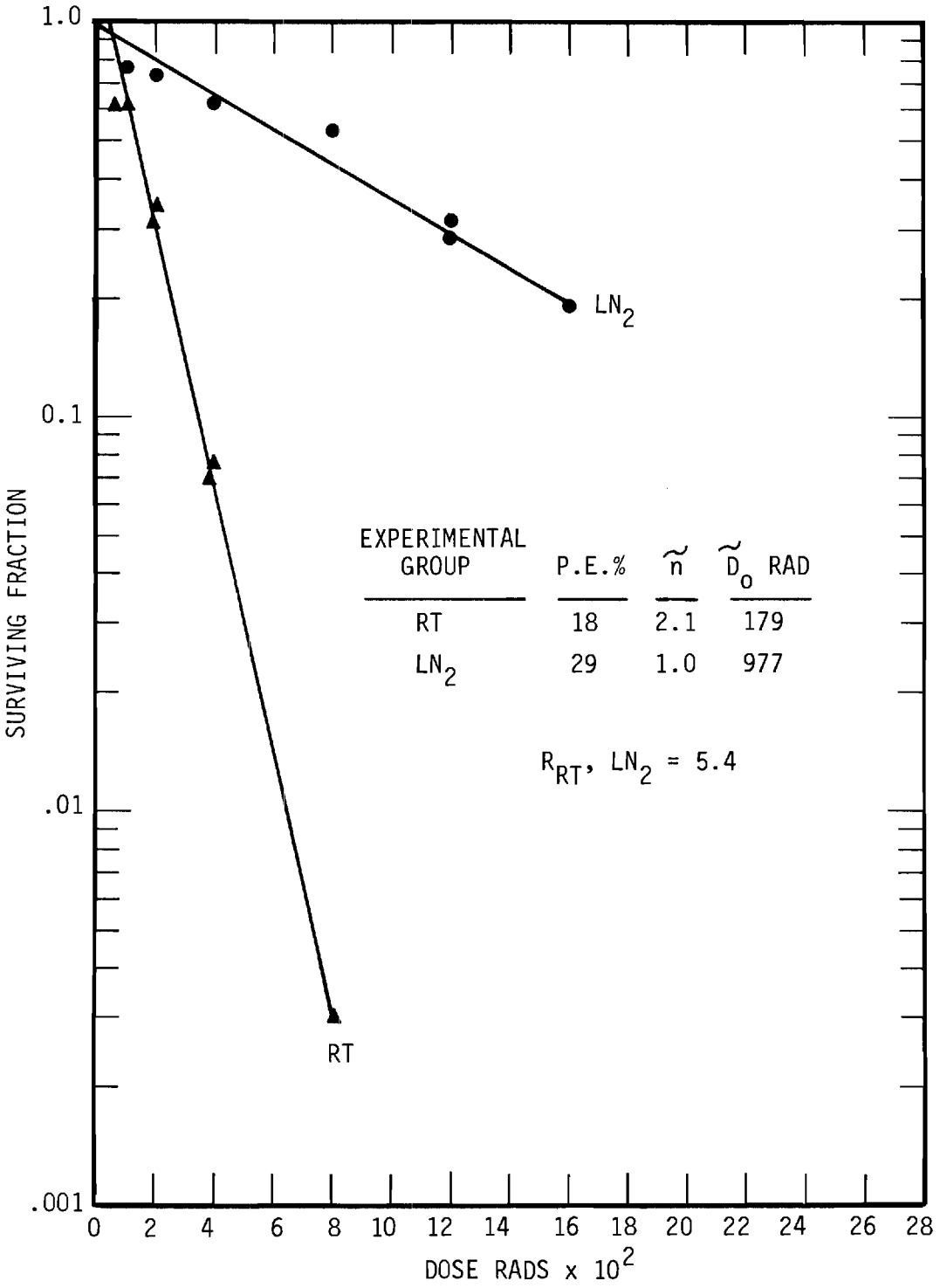

FIGURE 1. Reproductive survival of Chinese hamster cells as a function of 250 kV x-irradiation at room temperature and in liquid nitrogen

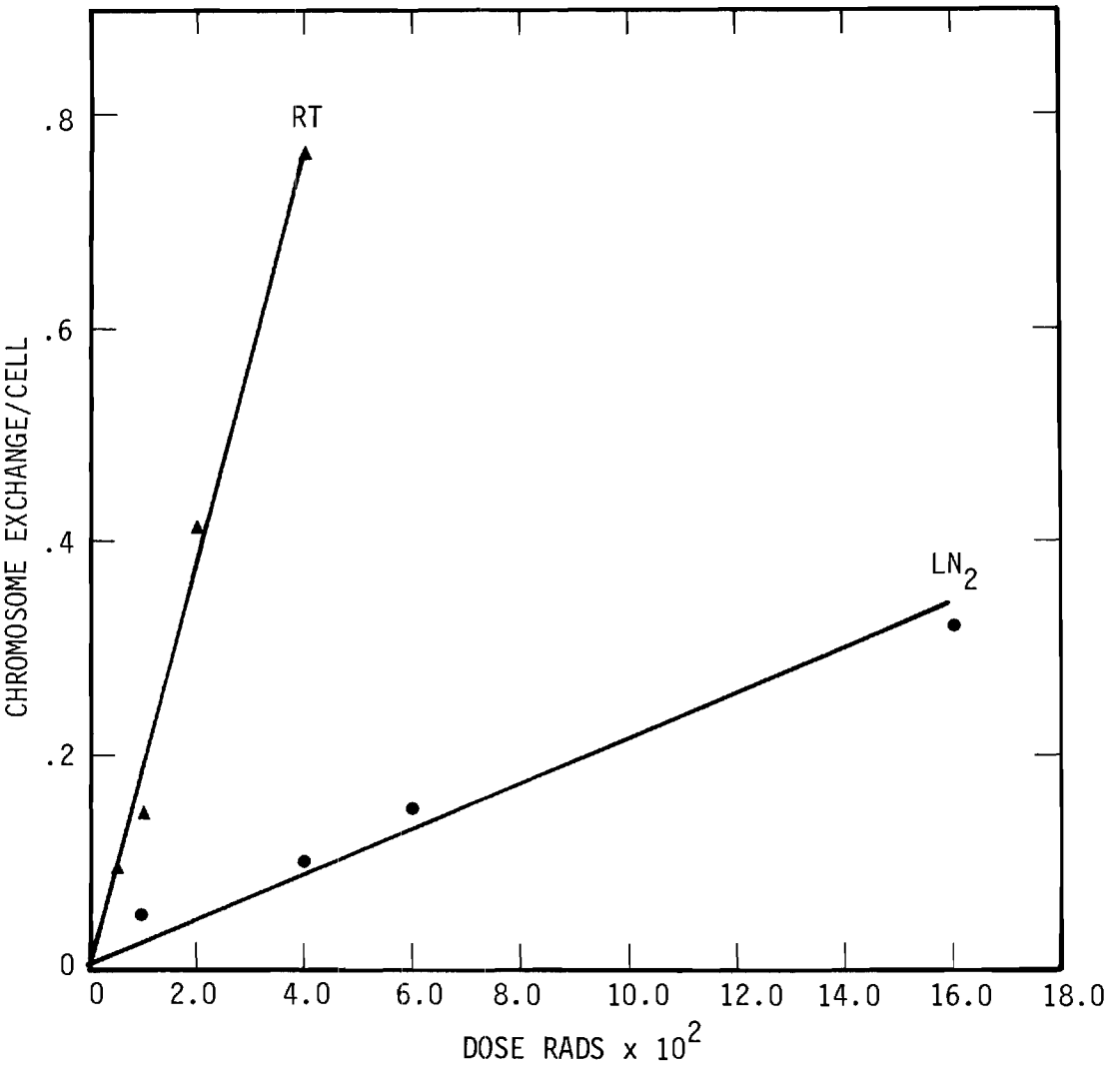

FIGURE 2. Frequency of chromosome exchanges produced in Chinese hamster cells with 250 kV x-rays at room temperature and in liquid nitrogen

where a and b are negative logarithms, with S log y·x = .0440 and r = .9804. The sample standard deviation of the regression coefficient (Sb) was equal to .0014 and a "t" test indicated that b was highly significant being 28 with 3 d.f.. The extrapolation number ($\tilde{n}$) was 1.0 and the dose required to reduce survival by 1/e ($\tilde{D}_o$) was 977 rads. Thus, irradiation in liquid nitrogen was $\frac{179}{977}$ of $\frac{1}{5.4}$ as effective in producing reproduction cell death as irradiation at room temperature in the same cell system.

Chromosome exchanges produced as a function of x-radiation at room temperatures are given in Table III and Figure 2. There appeared to be a linear relationship between chromosome aberration and dose with this material. A least squares fit gave the following relationship:

$$Y = .1909\ D(\text{rads} \times 10^2)$$

with $S_{y \cdot x} = .0362$.

The sample standard deviation of the regression coefficient (Sb) was .0144 and a "t" test indicated that b was highly significant being 13.3 with 2 degrees of freedom.

When cells were irradiated in liquid nitrogen, there was a sharp reduction in the frequency of chromosome exchanges. Table IV and Figure 2 give the results of these experiments. The frequency of exchanges as a function of dose was linear with these data and a least squares fit gave the following relationship:

$$Y = .0211\ D(\text{rad} \times 10^2)$$

with $S_{y \cdot x} = .00945$.

The sample standard deviation (Sb) was .000839 and a "t" test indicated

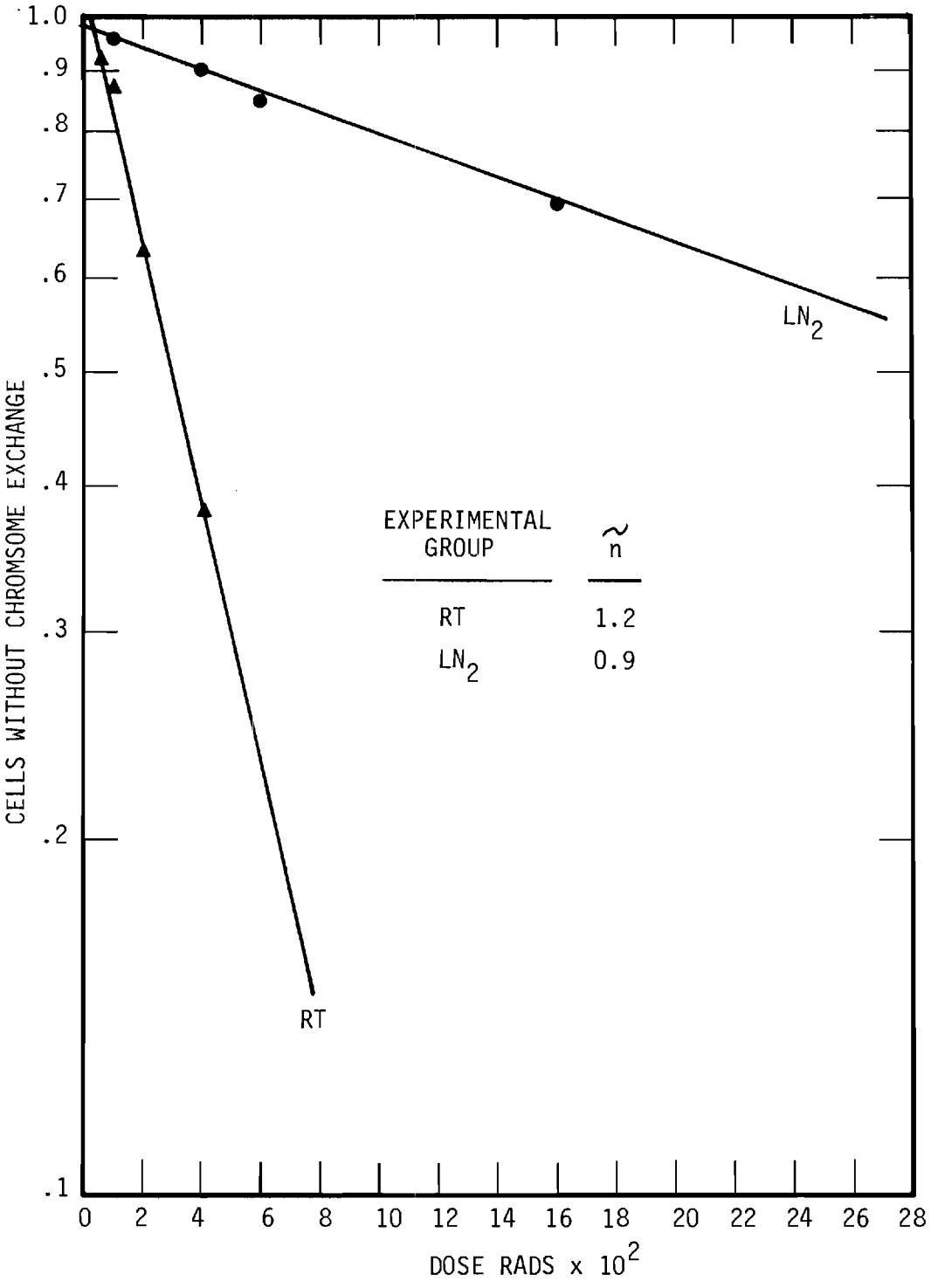

FIGURE 3. Frequency of cells without chromosome exchanges after various doses of 250 kV x-rays at room temperature and in liquid nitrogen

that b was highly significant being equal to 25.1 with 2 degrees of freedom.

Figure 3 presents the frequency of cells without chromosome aberrations as a function of dose at the two different temperatures.

Thus, at room temperature, chromosome exchanges were produced at a frequency of 190×10^{-5} per cell per rad and when irradiated in liquid nitrogen they were produced with a frequency of 21×10^{-5} per cell per rad. Radiation at liquid nitrogen temperatures was $\frac{21}{190}$ or $\frac{1}{9}$ as effective in producing chromosome exchanges as irradiation at room temperature. For irradiations performed at room temperature at the $\tilde{D}_o$, when there is enough radiation to cause reproductive cell death on the average in all of the cells, there are .34 exchanges per cell. At liquid nitrogen temperature, there are .21 exchanges per cell at the $\tilde{D}_o$. Thus, irradiation at very low temperatures results in a differentiated production of chromosome exchanges at the $\tilde{D}_o$ in cells. This may suggest that chromosome exchanges are not the primary cause of reproductive cell death after exposure to high energy radiation.

1. Articles in preparation

"Reproductive cell death and chromosome exchanges produced by 250 kV s-rays in Chinese hamster cells at room temperature and in liquid nitrogen."

DOSE RADS x 10^2	NUMBER OF CELLS SCORED (N)	CELLS WITHOUT ABERRATIONS (N_0)	NUMBER OF DICENTRICS (D)	NUMBER OF RINGS (R)	ABERRATION FREQUENCY PER CELL D+R/N	FREQUENCY OF CELLS WITHOUT ABERRATIONS N_0/N
.0	250	250	0	0	0	1.00
.5	120	110	9	2	.092 ± .027	.916
1.0	75	65	11	0	.147 ± .044	.866
2.0	58	36	20	4	.414 ± .084	.620
4.0	50	19	30	8	.760 ± .123	.380

TABLE III. Chromosome exchanges in Chinese hamster cells 20 hrs after various doses of 250 kV x-rays, administered at room temperature